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(54) Title: A TRYPTAMINE PRODUCING TRYPTOPHAN DECARBOXYLASE GENE OF PLANT ORIGIN

## (57) Abstract

Isolation and cloning of cDNA sequence of the tryptophan decarboxylase gene from *Catharanthus roseus* and the development of the cDNA sequence in a plasmid vector capable of transforming cell lines that will produce the tryptophan decarboxylase enzyme.

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# TITLE OF THE INVENTION

A tryptamine producing tryptophan decarboxylase gene of plant origin.

## BACKGROUND OF THE INVENTION

5           Tryptophan decarboxylase (TDC; E.C. 4.1.1.27) catalyses the conversion of L-tryptophan to tryptamine. This enzyme has been detected in numerous plant systems and it has been suggested that its primary role is to supply possible precursors for auxin biosynthesis (Baxter, C. & Slaytor, M. (1972) *Phytochemistry* 11, 10   2763-2766; Gibson, R.A., Barret, G. & Wightman F. (1972) *J. Exp. Bot.* 23, pages 775-786; Gross, W. & Klapchek, S. (1979) *Z. Pflanzenphysiol.* 93, pages 359-363).

          In the Gramineae, TDC catalyses the synthesis of precursors for the protoalkaloids which have 15   considerable physiological activity in higher animals (Smith, T.A., (1977) *Phytochemistry* Vol. 16, pages 171-175). It is also known that tryptophan-derived tryptamines are also precursors of the tricyclic  $\beta$ - 20   carboline alkaloids formed by condensation with a one- or two- carbon moiety (Slaytor, M., & McFarlane, I.J., (1968) *Phytochemistry* 7, pages 605-610).

          Furthermore, in periwinkle (Catharanthus roseus), TDC produces tryptamine for biosynthesis of the 25   commercially important antineoplastic monoterpenoid indole alkaloids, vinblastine and vincristine (De Luca, V., & Kurz, W.G.W. (1988), *Cell Culture and Somatic Cell Genetics of Plants*, Constabel, F. and Vasil, I.K., eds. Academic Press 5, pages 385-401).

30           The TDC from Catharanthus roseus has been purified to homogeneity. It occurs as a dimer consisting of 2 identical subunits of Mr 54,000 and it requires pyridoxal phosphate for activity (Noe, W., Mollenschott, C., & Berlin J. (1984) *Plant Mol. Biol.* 3, 35   pages 281-288).

          The enzyme possesses characteristics of plant aromatic decarboxylases which usually exhibit high

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substrate specificity. For example, TDC will decarboxylate L-tryptophan and 5-hydroxy-L-tryptophan but is inactive towards L-phenylalanine and L-tyrosine, while the tyrosine decarboxylases from Syringa vulgaris (Chapple, C.C.S., (1984) Ph.D. Thesis, University of Guelph, Guelph, Ontario, Canada), Thalictrum rugosum and Escholtzia californica (Marques, I.A., & Brodelius, P. (1988) Plant Physiol. 88, pages 52-55), accept L-tyrosine and L-dopa as substrates but not L-tryptophan or 5-hydroxy-L-tryptophan. The aromatic L-amino acid decarboxylases (dopa decarboxylase (DDC), ED 4.1.1.28) of D. melanogaster (Clark, W.C., Pass, P.S., Venkataraman, B., & Hodgetts, R.B. (1978) Mol. Gen. Genet. 162, pages 287-297; Eveleth, D.D., Gietz, R.D., Spencer C.A., Nargang, F.E., Hodgetts, R.B. & Marsh, J.L. (1986) Embo. J. 5, pages 2663-2672; Morgan B.A., Johnson, W.A. & Hirsh, J. (1986) Embo. J. 5, pages 3335-3342) and mammals (Albert, V.R., Allen, J.M., & Joh, T.H. (1987) J. Biol. Chem. 262, pages 9404-9411) have a broader substrate specificity with L-dopa, tyrosine, phenylalanine and possibly histidine also serving as substrates.

In animals, the role of aromatic L-amino acid decarboxylase is to produce the major neurotransmitters dopamine and serotonin and, in D. melanogaster, the DDC enzyme serves a second, inducible role, in the sclerotization of the insect cuticle (Christenson, J.G., Dairman, W. & Udenfriend, S. (1972) Proc. Natl. Acad. Sci. USA 69, pages 343-347; Lovenberg, W., Weissbach, W., & Udenfriend S. (1962) J. Biol. Chem. 237, pages 89-93; Yuwiler, A., Geller, E. & Eiduson, S. (1954) Arch. Biochem. Biophys. 80, pages 162-173; Brunet, P. (1980) Insect Biochem. 10, pages 467-500).

It would appear highly desirable to be able to clone the cDNA sequence of tryptophan decarboxylase from Catharanthus roseus, thus, providing the development of the cDNA sequence in a plasmid vector capable of

transforming cell lines that will produce the tryptophan decarboxylase enzyme.

5 If the tryptophan decarboxylase gene could be inserted into living organisms by transformation to produce tryptamine and related protoalkaloids, it could supplement a neurotransmitter deficiency.

10 Further, the insertion of this gene in plants could be useful to alter the spectrum of tryptophan-based chemicals normally produced by the plant. For example, the insertion of constitutive expression of tryptophan decarboxylase in Brassica species could sequester the cytoplasmic tryptophan pool for the synthesis of tryptamine and related protoalkaloids and therefore repress the normal synthesis and accumulation  
15 of indole glucosinolates.

Hence, creation of plants with an altered chemical spectrum may produce novel phenotypes which have resistance to various pathogenic diseases or to insect pests.

## 20 SUMMARY OF THE INVENTION

In accordance with the present invention, there is now provided the sequence of a cDNA clone which includes the complete coding region of tryptophan decarboxylase, preferably tryptophan decarboxylase (E.C.  
25 4.1.1.27) from periwinkle (Catharanthus roseus). The cDNA clone (1747 bp) was isolated by antibody screening of a cDNA expression library produced from poly A<sup>+</sup> RNA found in developing seedlings of C. roseus. The clone hybridized to a 1.8 kb mRNA from developing seedlings  
30 and from young leaves of mature plants.

Also within the scope of the present invention is a method for inserting TDC gene into living organisms by transformation. The identity of the clone was confirmed when extracts of transformed E. coli expressed  
35 a protein containing tryptophan decarboxylase enzyme activity. The tryptophan decarboxylase cDNA clone encodes a protein of 500 amino acids with a calculated

molecular mass of 56,142 Da. The amino acid sequence shows a high degree of similarity with the aromatic L-amino acid decarboxylase (dopa-decarboxylase) and the alpha-methyldopa hypersensitive protein of Drosophila  
5 melanogaster. The tryptophan decarboxylase sequence also showed significant similarity to feline glutamic acid decarboxylase and mouse ornithine decarboxylase suggesting a possible evolutionary link between these amino acid decarboxylases.

10 Furthermore, the protein encoded by the cDNA clone of the present invention is active in vitro.

#### IN THE DRAWINGS

Figure 1 (lane 2) represents the TDC enzymatic activity in extracts of pTDC5-transformed E. coli,  
15 compared to those in control E. coli (lane 1) and that in C. roseus itself (lane 3).

Figure 2 represents the hybridization of the pTDC-5 clone to a 1.8 kb mRNA species isolated from periwinkle.

20 Figure 3 shows the nucleotide sequence of the pTDC5 cDNA clone and its deduced amino acid sequence. The putative polyadenylation signal is underlined.

Figure 4 shows the amino acid sequence alignments of the protein for the D. melanogaster alpha  
25 methyldopa hypersensitive gene (AMD), C. roseus tryptophan decarboxylase (TDC), and Drosophila DOPA decarboxylase isoenzyme 1 (DDC1).

Figure 5 shows hydropathy profile of TDC and DDC1.

30 Other advantages of the present invention will be readily illustrated by referring to the following description.

#### DETAILED DESCRIPTION OF THE INVENTION

cDNA synthesis and DNA sequencing.

35 Seedlings of C. roseus (L.) G. Don cv "Little Delicata" were germinated and grown for 5 days in the dark as described previously (De Luca, V., Alvarez-

Fernandez, F., Campbell, D., & Kurz, W.G.W. (1988) *Plant Physiol.* 86, 447-450). Seedlings were harvested after 18 hours of light treatment and total RNA was isolated as described by Jones, J.D.G., Dunsmuir, P. & Bedrook, J. (1985) *EMBO J.* 4, 2411-2418.

Poly(A)<sup>+</sup> RNA was isolated by chromatography on oligo (dT)<sup>-</sup> cellulose (Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* 69, 1408-1412) and double-stranded cDNAs were prepared according to the procedure of Gubler and Hoffman (1983, *Gene* 25, 263-269). Following ligation with Eco RI linker, the cDNA was inserted into the Eco RI site of the expression vector ZAP (Stratagene, San Diego, Short, J.M., Fernandez, J.M., Sorge, J.A. & Huse, W.D. (1988) *Nucl. Acids Res.* 16, 7583-7600). A library containing 3.1 X 10<sup>5</sup> recombinant phages was obtained and after amplification, 2 X 10<sup>5</sup> plaques were screened with specific polyclonal antiserum raised against-TDC. Plasmids (pBluescript) containing a TDC cDNA insert were rescued using the R408 fl helper phage (Short, J.M., Fernandez, J.M., Sorge, J.A. & Huse, W.D. (1988) *Nucl. Acids Res.* 16, 7583-7600) and the nucleotide sequence of a full-length cDNA clone (pTDC5) was determined on both strands by the dideoxy-chain-termination method (Sanger, F., Nicklen, S. & Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463-5467). The sequencing strategy included subcloning of restriction fragments and the use of oligonucleotide primers. The sequence for all restriction sites used for the subcloning was determined on at least one strand. Comparisons of the pTDC5 cDNA nucleotide sequence and of the deduced amino acid sequence with Genbank and NBRF sequence libraries were performed using the FASTA program package (Pearson, W.R. & Lipman, D.J. (1988) *Proc. Natl. Acad. Sci. USA* 85, 2444-2448).

RNA blot hybridization.

Poly(A)<sup>+</sup> RNA was isolated from 6 day old developing seedlings and from young leaves of mature

plants as described above. These tissues were chosen as a likely source of TDC poly(A)<sup>+</sup> RNA based on the presence of high levels of TDC enzyme activity (De Luca, V., Alvarez-Fernandez, F., Campbell, D., & Kurz, W.G.W. (1988) Plant Physiol. 86, 447-450). RNA was denatured, fractionated by electrophoresis on formaldehyde/agarose gels, and then transferred to nitrocellulose filters (Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982) In: Molecular Cloning, A Laboratory Manual. Cold Spring Harbor, New York). Blotted RNA was hybridized to [<sup>32</sup>P]-labelled pTDC5 DNA and autoradiography was performed using Kodak XAR-5 films.

TDC activity in extracts of E. coli.

A culture (100 ml) of the E. coli strain ZL1-blue containing pTDC5 or pBluescript was incubated at 37°C for 2 hours before adding the IPTG inducer at a final concentration of 1 mM. Incubation was continued for an additional 2 hours. Cells were harvested, washed in TE buffer, resuspended and lysed in 3 ml of a buffer containing 0.1 M Hepes, pH 7.5, 1 mM DTT. Debris was removed by centrifugation and the supernatant was desalted by passage over a Sephadex G-25<sup>th</sup> column. TDC enzymatic activity in bacterial supernatants was determined by monitoring the conversion of L-[methylene-<sup>14</sup>C]-tryptophan to [<sup>14</sup>C]-tryptamine (De Luca, V., Alvarez-Fernandez, F., Campbell, D., & Kurz, W.G.W. (1988) Plant Physiol. 86, 447-450). Supernatants (30 µl) were incubated in the presence of 0.1 µCi of [<sup>14</sup>C]-tryptophan (sp. act. 59 mCi/mmol.) for 30 minutes and reactions were stopped with 100 µl NaOH. Radioactive tryptamine was extracted from the reaction mixture with ethyl acetate and was analyzed by silica gel thin layer chromatography and autoradiography. Determination of TDC enzyme activity in leaves was performed as described previously (De Luca, V., Alvarez-Fernandez, F., Campbell, D., & Kurz, W.G.W. (1988) Plant Physiol. 86, 447-450).

TDC enzymatic activity in E. coli.

5 A tryptophan decarboxylase cDNA clone of C. roseus was isolated by the use of antibody screening of an expression library. The antigenicity and enzymatic activity (Figure 1) of the encoded protein established the identity of the TDC cDNA.

10 When the original cDNA library was screened with the anti-TDC antibody, 27 clones were identified. Six clones were selected and submitted to further analysis. Partial sequence analysis revealed no difference among these clones, except for their length. Therefore, the clone having the longest cDNA insert (pTDC5) was selected for further characterization. To confirm that this cDNA clone corresponded to TDC, enzymatic activity  
15 was measured in cell extracts from E. coli. Figure 1 shows that [<sup>14</sup>C]-tryptamine was produced with extracts from cells transformed with pTDC5, and with extracts from C. roseus leaves (lane 3), but not with extracts from cells containing only the vector (lane 1).

20 The conversion of [<sup>14</sup>C]-tryptophan to [<sup>14</sup>C]-tryptamine was monitored in extracts of E. coli and C. roseus leaves. [<sup>14</sup>C]-tryptophan (sp. act. 50 mCi/mmol) for 30 minutes. After addition of base, [<sup>14</sup>C]-tryptamine was extracted from the reaction mixture with ethyl acetate and reaction products were analyzed by thin  
25 layer chromatography on silica gel (solvent CHCl<sub>3</sub> MeOH: 25% NH<sub>3</sub> (5:4:1) and autoradiography. In Figure 1, TDC enzymatic activity is shown; lane 1, E. coli is transformed by the pBluescript vector, lane 2, E. coli is transformed by pTDC5 and lane 3, C. roseus extract is shown.  
30

35 This result indicated that TDC enzymatic activity was retained by the protein produced using a TDC cDNA clone under the control of the Lac promoter of the pBluescript vector. No attempts were made to quantify the level of activity of TDC in E. coli.

## Sequence analysis of a TDC cDNA clone.

DNA sequence analysis of pTDC5 revealed the presence of an open reading frame coding for a protein of 500 amino acids, which corresponded to a molecular mass of 56,142 Da (Figure 2). The 5'-nontranslated region of pTDC5 contained 69 nucleotides and included, near its beginning, a long stretch of alternating pyrimidines. Sequence around the methionine initiation codon (AAUAAUGGG) matched closely the consensus sequence for plant gene initiation codons (AACAAUGGC) (Lütcke, H.A., Chow, K.C., Mickel, F.S., Moss, K.A., Kerm, H.F. and Scheele, G.A. (1987) EMBO J. 6, 43-48). The 3'-nontranslated region consisted of 168 nucleotides up to the poly(A) tail and contained an AAUAAA putative poly(A)<sup>+</sup> addition signal 17 nucleotides upstream from the start of the poly(A)<sup>+</sup> tail. Examination of the predicted amino acid sequence did not reveal the presence of a signal sequence (Watson, M.E.E. (1984) Nucl. Acids Res. 12, 5145-5164), which is consistent with the proposed cytoplasmic location of TDC within the cell (De Luca, V., Alvarez-Fernandez, F., Campbell, D., & Kurz, W.G.W. (1988) Plant Physiol. 86, 4474-50).

Comparison of TDC-cDNA nucleotide and deduced amino acid sequences with nucleotide sequences in the Genbank DNA sequence database and with amino acid sequences in the NBRF protein sequence database revealed surprising similarity (40% amino acid identity) with the dopa-decarboxylase isoenzyme 1(DDC1) from D. melanogaster (Eveleth, D.D., Gietz, R.D., Spencer, C.A., Nargang, F.E., Hodgetts, R.B. & Marsh, J.L. (1986) EMBO J. 5, 2663-2672; Morgan, B.A., Johnson, W.A. & Hirsh, J. (1986) EMBO J. 5, 3335-3342), and with the protein corresponding to the D. melanogaster alpha-methyl dopa hypersensitive gene (AMD, 35% amino acid identity) (Eveleth, D.D. & Marsh, J.L. (1986) Genetics 114, 469-483) (Figure 3). In Figure 3, the boxes show TDC residues present in AMD and/or DDC1 sequences. Amino

acids are numbered for TDC (top) and DDC1 (bottom). The areas of amino acid similarity extended throughout the protein and were not restricted to a particular portion of either structure.

5 Furthermore, the 39% amino acid sequence similarity could be extended to the predicted distribution of potential alpha helices and beta sheets. This indicated that the amino acid differences between the two proteins did not significantly alter their  
10 secondary structures, and may indicate the importance of such conserved domains to mediate subunit assembly, as well as catalytic function and substrate specificity.

Limited proteolysis of pig kidney dopa decarboxylase and amino acid sequencing of a tryptic  
15 fragment produced a sequence for 50 amino acid residues one third of the distance from the COOH terminus of this protein. (Tancini, B., Dominici, P., Simmaco, M., Schinina, M.E., Barra, D., & Voltatormi, C.D. (1988) Arch. Biochem. Biophys. 260, 569-576). Comparison of this 50 amino acid sequence with periwinkle TDC and D. melanogaster DDCI gave 20 and 32 identical amino acids,  
20 respectively. Furthermore, comparison of C. roseus TDC to feline glutamic acid decarboxylase (Kobayashi, Y., Kaufman, D.L. & Tobin, A.J. (1987) J. Neurosci. 7, 2768-2772) showed that 10% of the amino acid residues were  
25 identical between these two proteins. This similarity could be extended to 25% on a 396 aa stretch. Mouse ornithine decarboxylase (Kahana, C. & Nathans, D. (1985) Proc. Natl. Acad. Sci. USA 82, 1673-1677) showed a statistically significant (Pearson, W.R. & Lipman, D.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2444-2448) 12%  
30 amino acid sequence similarity to the plant TDC which also extended throughout the protein sequence. We also found that the sequence Pro-His-Lys, beginning at  
35 position 317 in TDC, was identical to the sequence at the pyridoxal phosphate binding sites of D. melanogaster DDC (Marques, I.A., & Brodelius, P. (1988) Plant

Physiol. 88, 52-55; Clark, W.C., Pass, P.S., Venkataraman, B., & Hodgetts, R.B. (1978) Mol. Gen. Genet. 162, 287-297), feline glutamic acid decarboxylase (Kobayashi, Y., Kaufman, D.L. & Tobin, A.J. (1987) J. Neurosci. 7, 2768-2772) and pig dopa-decarboxylase (Bossa, F., Martini, F., Barra, D., Borri Voltatorni, C., Minelli, A. & Turano, C., (1977) Biochem. Biophys. Res. Commun. 78, 177-183). In contrast, the AMD protein, whose enzymatic function is unknown, contained the sequence Leu-His-Lys at the pyridoxal phosphate binding domain. The sequence similarity observed between TDC, feline glutamic acid decarboxylase and mouse ornithine decarboxylase also suggests an evolutionary link between these three amino acid decarboxylases.

Structural similarities between TDC and D. melanogaster DDC1 proteins were further revealed by comparing their hydropathy profiles (Figure 4). Each value was calculated as the average hydropathic index of a sequence of 9 amino acids and plotted to the middle residue of each sequence. Positive and negative values indicate hydrophobic and hydrophilic regions of the proteins, respectively. Close examination of the alignment of hydrophobic and hydrophilic regions for the two proteins showed a striking match between them, except for the area near the N terminus and the region around TDC residue 225.

Most decarboxylases require for their activity a pyridoxal phosphate co-factor linked to the C amino group of a lysine residue. The observed similarities around the pyridoxal binding site of pig kidney dopa-decarboxylase, D. melanogaster dopa-decarboxylase and feline glutamate decarboxylase with that of periwinkle TDC strongly suggests that lysine 319 of TDC binds pyridoxal phosphate.

The aromatic amino acid decarboxylases of plants, insects and mammals are remarkably similar in

subunit structure, molecular mass and kinetic properties (Maneckjee, R., & Baylin, S.B. (1983) *Biochemistry* 22, 6058-6063). Plant aromatic amino acid decarboxylases (Noe, W., Mollenschott, C. & Berline J. (1984) *Plant Mol. Biol.* 3, pages 281-288; Chapple, C.C.S., (1984) Ph.D. Thesis, University of Guelph, Guelph, Ontario, Canada; Marques, I.A., & Brodelius, P. (1988) *Plant Physiol.* 88, pages 52-55), in contrast to those from animals, display high substrate specificity for indole or phenolic substrates but not to both. The strong similarity observed between periwinkle TDC and DDC1 of *D. melanogaster* suggests that plant aromatic amino acid decarboxylase specific for tyrosine, phenylalanine or dihydroxyphenylalanine may be structurally similar to TDC and may, therefore, also be evolutionarily related. The recent purification of specific L-tyrosine decarboxylases (Marques, I.A., & Brodelius, P. (1988) *Plant Physiol.* 88, pages 52-55) to homogeneity should allow cloning of these genes and direct testing of this hypothesis.

#### TDC mRNA accumulation.

Total poly(A)<sup>+</sup> RNAs (1 µg) from six day old *C. roseus* seedlings and from young leaves of mature plants were run on an agarose/formaldehyde gel and were transferred to nitrocellulose paper. Hybridization was performed with [<sup>32</sup>P]-labelled pTDC5 insert (sp. act. 1.2 X 10<sup>8</sup> cpm/µG). When total poly(A)<sup>+</sup> RNA isolated from six day old seedlings was probed with a 1.6 kb cDNA fragment isolated from pTDC5, a 1.8 kb mRNA was detected (Figure 5, lane 1). Young leaves from the mature plant also contained a 1.8 kb mRNA (Figure 5, lane 2). A fainter signal corresponding to a transcript of 3.2 kb was also present in both the lanes. This signal could be a precursor form of the TDC mRNA or an unrelated transcript having some sequence similarity to TDC.

CLAIMS

1. A DNA sequence comprising the cDNA sequence of the tryptophan decarboxylase gene.
- 5 2. The DNA sequence as defined in claim 1, wherein the tryptophan decarboxylase gene is closed and sequenced from Catharanthus roseus.
3. A synthetic recombinant DNA molecule containing a DNA sequence comprising the cDNA sequence of the tryptophan decarboxylase gene.
- 10 4. A synthetic recombinant DNA molecule as defined in claim 3, wherein the tryptophan decarboxylase gene is cloned and sequenced from Catharanthus roseus.
5. A synthetic DNA molecule expressible in E. coli and coding for the expression of the tryptophan decarboxylase enzyme.
- 15 6. The synthetic DNA molecule of claim 5, wherein the tryptophan decarboxylase gene is from Catharanthus roseus.
- 20 7. An expression vector comprising a synthetic DNA molecule coding for the tryptophan decarboxylase enzyme.
8. An expression vector having a microorganism replication system and a gene coding for the expression of the tryptophan decarboxylase enzyme.
- 25 9. The expression vector of claim 8, wherein the microorganism is E. coli and wherein the tryptophan decarboxylase enzyme is from Catharanthus roseus.
10. A host cell having an extrachromosomal functional synthetic gene expressing an active tryptophan decarboxylase enzyme.
- 30 11. A cell according to claim 10, wherein said cell is a microorganism and wherein said tryptophan decarboxylase enzyme is from Catharanthus roseus.
- 35 12. A cell according to claim 11, wherein said microorganism is a bacterium.

-13-

13. A cell according to claim 11, wherein said bacterium is E. coli.

14. An E. coli bacteria having an extrachromosomal functional synthetic gene expressing an active tryptophan decarboxylase enzyme.

5

1/6

1

2

3



FIG. 1

1

2

1.8 ▶

FIG. 2

SUBSTITUTE SHEET



3/6

ACCAGAAAAAGAAAAAATA  
 lys pro leu glu ala glu met gly ser ile asp ser thr  
 AAG CCA CTT GAA GCT GAG ATG GGC AGC ATT GAT TCA ACA  
 val glu thr tyr pro val glu phe arg lys gln ala his  
 GTG GAA ACA TAT CCG GTC CTT AGC GAA GTC GAA pro gly  
 leu pro glu pro leu asp asp ile met lys asp ile gln  
 CTC CCC GAA CCA CTT GAC GAC ATC ATG AAA GAT ATT CAG  
 pro asn phe tyr ala phe phe pro ala thr val ser ser  
 CCT AAT TTT TAT GCA TTT TTT CCT GCC ACT GTT AGT TCA  
 asn ser val gly phe thr trp val ser ser pro ala ala  
 AAT TCA GTA GGC TTT ACT TGG GTT TCT TCA CCA GCC GCC  
 gln ile leu lys leu pro lys ser phe met phe ser gly  
 CAG ATC CTT AAA CTC CCC AAA TCT TTC ATG TTT TCA GGT  
 ser ile leu cys thr ile ile ala ala arg glu arg ala  
 TCC ATT CTT TGT ACA ATC ATT GCC GCC CGG GAA AGG GCC  
 val cys tyr gly ser asp gln thr his thr met phe pro  
 GTC TGT TAC GGA TCC GAT CAA ACC CAT ACC ATG TTC CCC  
 ile arg leu ile pro thr thr val glu thr asp phe gly  
 ATT AGG TTA ATA CCT ACG ACC GTC GAA ACG GAT TTC GGC  
 asp val ala ala gly tyr val pro leu phe leu cys ala  
 GAC GTG GCG GCC GGA TAT GTA CCG CTG TTC TTA TGC GCT  
 val asp ser leu ser glu ile ala asn glu phe gly ile  
 GTG GAC TCA CTT TCT GAA ATC GCT AAC GAG TTT GGT ATT  
 cys ile cys pro glu phe arg his tyr leu asp gly ile  
 TGT ATA TGT CCC GAG TTT AGA CAT TAC TTG GAT GGA ATC  
 trp leu leu ala tyr leu asp cys thr cys leu trp val  
 TGG CTA CTC GCT TAC TTA GAT TGC ACT TGC TTG TGG GTC  
 asn pro glu tyr leu lys asn lys gln ser asp leu asp  
 AAT CCT GAG TAT TTA AAA AAT AAA CAG AGT GAT TTA GAC  
 gly arg lys phe arg ser leu lys leu trp leu ile leu  
 GGA CGA AAA TTT CCG TCG CTG AAA CTT TGG CTC ATT TTA  
 arg ser asp val ala met gly lys met phe glu glu trp  
 CGT TCT GAC GTC GCA ATG GGC AAA ATG TTC GAA GAA TGG  
 arg asn phe ser leu val cys phe arg leu lys pro asp  
 AGA AAC TTT TCT CTT GTT TGT TTT AGA TTA AAA CCT GAC  
 leu leu asp met leu asn ser thr gly arg val tyr met  
 CTT TTG GAC ATG CTT AAC TCG ACG GGA CGA GTT TAT ATG  
 leu ala val gly ser ser leu thr glu glu his his val  
 CTG GCT GTT GGC TCA TCG CTA ACT GAA GAA CAT CAT GTA  
 asp leu leu lys glu ala ter TGA TGAATAAGTAAGGGTTTTTTTTTA  
 GAT TTG CTC AAA GAA GCT TGA  
 TAAAGTGATTTGTAAAGGTTTATTGTACTCAAACAATCATGCAATTAATTAT  
 AAAA

  
 (CONT.)

SUBSTITUTE SHEET

4/6

AMD  
TDC  
DDC1

10 20 30 40 50 60

MGSTIDSTNVAMNSPVGEFKPL-----MDAKREFREFGKAAIDYIADYLENIR  
MSHIPISNTIPTKTQDGNCKANISPDKLDPKVSIIDMEAPFEKDFAKIMVDFIAYYLENIR

AMD  
TDC  
DDC1

50 60 70 80 90 100 110 120

DDDVLPNVEPGYLLDLPTTEPFEPAWQDVLCDISRVITKPGLIHSESHMHAYIPTSTS  
TYPVLSEVEPGYLRKRIPETAPYLPPEPLDDIMKDIQKDIIPGMTNMTSPNFYAFFPATVS  
ERRVLPFEVKPGYLRKPLIPDAAPEKPEKWQDMQDIERVIMPGVITHMHSKETHAYFPPTANS

AMD  
TDC  
DDC1

110 120 130 140 150 160 170 180

YPSIVGEMLASGFGVIGFNTICSPACTELEVVVMDWLAQILKLPKSFHQAHSDFGCGGVIO  
SAPFLGEMLSIALNSVGFTWVSSPAATELEMIIVMDWLAQILKLPKSFHQAHSDFGCGGVIO  
YPAYVADMLSGAIACIGFTWIASPACTELEVVVMDWLGKMLPLPAEFLACSGKGGGVIO

AMD  
TDC  
DDC1

170 180 190 200 210 220 230 240

GSASEAVLVAVLAAREQAIANYRESHPEL-SESEVRGLVAYSSDQSNSCIERRAGVLAAM  
NTISESLCTIIAAREPALE-----KLGPDISIGKLVCYGSDQHTMTMFPKTKLAGI  
GTASESLVASAGSQGQVEVEGEGAPSGVGLHTILGKLVGYCSDQAHSSVERAGLGGV

AMD  
TDC  
DDC1

220 230 240 250 260 270 280 290

---PIRLLPA--GEDEVLRGDTLRGAIEDVAAAGRIIPVICVATLGTGICAYDDIESLSA  
YPNNIRLIPITTVETDEGISPOVLRKMVEDDVAAGYVPLFLCATLGTGTTTIAIDPVDLSLSE  
---KLRVQSENHR---MRGAALERAIEDQVAEGLIPFYAVVTLGTNTSCAFDYIDECEGP

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AMD 280 VCEFEKCGSMLPMRMRWSLCSGGMF-GFAKGI GSRGLAFQPAQVHAGQRLRLGHVARGC 330  
 TDC IANEEGIIWIHVDAAYAGSACICPEFRHYLDGIERVDSLSLSPHKWLLAYLDC TCI MVKQP  
 DDC1 VGNKHNIWIHVDAAYAGSAFICPEYRHLMKGIESADSFNFPHKWL VNFDCSAMLKDP 350

AMD 340 QQGGRQLCCGSHLSEAQARGSVANSRLPSLANPLGRFRALRKMITERLEAEGRLNHVA 390  
 TDC HLLLRALTNNPEYLNKQSDLDKVVDFKNWQIATGRKFRSLKLWLILRSYGVVNLQSHIR  
 DDC1 SWVNVNENFVNDPELYLNKQSDLDKVVDFKNWQIATGRKFRSLKLWLILRSYGVVNLQSHIR 410

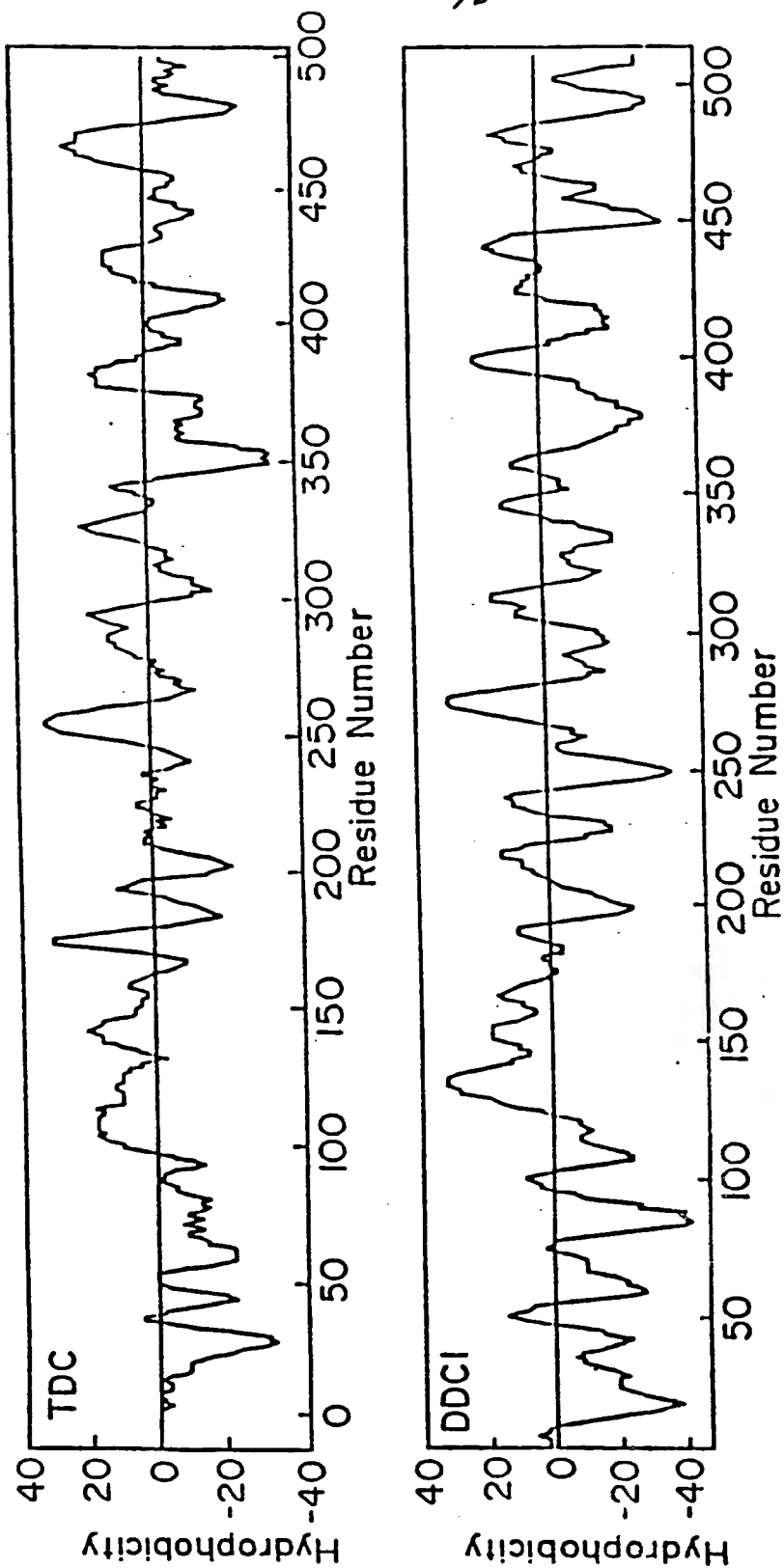
AMD 400 KHIELAKQFEQIVLNDSRFELVAPRALGLVCFRPPKGD-----NEITQLLQRLMDTKKV 450  
 TDC SDVAMGKMFEEWVRSDSRFEIVVPRNFSLVCFRLKPDVSSLHVEEYNNKKLLDMLNLTGRV  
 DDC1 RHCNFAKQFGCDLCVADSRFELAAEINMGLVSFRLLKGS-----NERNEALLKRINGRCHI 460

AMD 460 YMTKTEHAGROFLRFVVC GMDTKASDIDFAMQEI ESQLTDLQADESLVARKSGNVGDLAH 500  
 TDC YMTHTIVGCTYMLRLAVGSSITEEHHVRRVMDLIQKLTDDLLKEA  
 DDC1 HLVPAKIKDVYGLRLMAICSRFTQSEDMEYSWKEVSAADEMECEQ 510

AMD DFQIHLSTENATHEKSQ

 4  
 (CONT.)

SUBSTITUTE SHEET

FIG. 5

SUBSTITUTE SHEET

# INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 90/00057

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>5</sup> : C 12 N 15/60, 1/21, 9/88, // (C 12 N 1/21, C 12 R 1:19)														
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched †</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border-bottom: 1px solid black;">Classification System</th> <th style="width: 75%; border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border-right: 1px solid black; padding: 5px; vertical-align: top;">IPC<sup>5</sup></td> <td style="padding: 5px; vertical-align: top;">C 12 N</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ‡</div>			Classification System	Classification Symbols	IPC <sup>5</sup>	C 12 N								
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<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT †</b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 5px;">Category *</th> <th style="width: 70%; padding: 5px;">Citation of Document, †† with indication, where appropriate, of the relevant passages †‡</th> <th style="width: 20%; padding: 5px;">Relevant to Claim No. †‡</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">           Plant Molecular Biology, no. 3, 1984,            Martinus Nijhoff/Dr W. Junk Publishers,            (Dordrecht, NL),            W. Noé et al.: "Tryptophan            decarboxylase from Catharanthus            roseus cell suspension cultures:            purification, molecular and kinetic            data of the homogenous protein,            pages 281-288            see the whole document            cited in the application            --         </td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-14</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">           EMBO Journal, volume 5, no. 10, 1986,            IRL Press Limited, (Oxford, GB),            D.D. Eveleth et al.: "Sequence and            structure of the dopa decarboxylase            gene of Drosophila: evidence for            novel RNA splicing variants",            see pages 2664-2668            cited in the application            --         </td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-14</td> </tr> <tr> <td colspan="2" style="text-align: right; padding: 5px;">./.</td> <td></td> </tr> </tbody> </table>			Category *	Citation of Document, †† with indication, where appropriate, of the relevant passages †‡	Relevant to Claim No. †‡	Y	Plant Molecular Biology, no. 3, 1984, Martinus Nijhoff/Dr W. Junk Publishers, (Dordrecht, NL), W. Noé et al.: "Tryptophan decarboxylase from Catharanthus roseus cell suspension cultures: purification, molecular and kinetic data of the homogenous protein, pages 281-288 see the whole document cited in the application --	1-14	Y	EMBO Journal, volume 5, no. 10, 1986, IRL Press Limited, (Oxford, GB), D.D. Eveleth et al.: "Sequence and structure of the dopa decarboxylase gene of Drosophila: evidence for novel RNA splicing variants", see pages 2664-2668 cited in the application --	1-14	./.		
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: †§</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"G" document member of the same patent family</p> </div> </div>														
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">           Date of the Actual Completion of the International Search  <div style="text-align: center; border-top: 1px solid black; padding-top: 5px;">16th May 1990</div> </td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">           Date of Mailing of this International Search Report  <div style="text-align: center; border-top: 1px solid black; padding-top: 5px;">15. 06. 90</div> </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">           International Searching Authority  <div style="text-align: center; border-top: 1px solid black; padding-top: 5px;">EUROPEAN PATENT OFFICE</div> </td> <td style="border-bottom: 1px solid black; padding: 5px;">           Signature of Authorized Officer  <div style="display: flex; align-items: center; justify-content: center; padding-top: 5px;"> <div style="text-align: center; margin-right: 20px;"> </div> <div style="border: 1px solid black; padding: 2px 10px;">M. PEIS</div> </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center; border-top: 1px solid black; padding-top: 5px;">16th May 1990</div>	Date of Mailing of this International Search Report <div style="text-align: center; border-top: 1px solid black; padding-top: 5px;">15. 06. 90</div>	International Searching Authority <div style="text-align: center; border-top: 1px solid black; padding-top: 5px;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="display: flex; align-items: center; justify-content: center; padding-top: 5px;"> <div style="text-align: center; margin-right: 20px;"> </div> <div style="border: 1px solid black; padding: 2px 10px;">M. PEIS</div> </div>								
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## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	<p>Progress in Catecholamine Research, Part A: Basic Aspects and Peripheral Mechanisms, 1988, Alan R. Liss, Inc., Tong H. Joh et al.: "Molecular biology of Aromatic L-amino acid decarboxylase and dopamine beta-hydroxylase", see pages 30-31</p> <p>--</p>	1-14
A	<p>Journal of Biological Chemistry, volume 262, no. 19, 5 July 1987, The American Society of Biological Chemists, Inc., (US), V.R. Albert et al.: "A single gene codes for aromatic L-amino acid decarboxylase in both neuronal and non-neuronal tissues", pages 8404-9411 see page 9406</p> <p>--</p>	1-14
P,X	<p>Procl. Natl. Acad. Sci. USA, volume 86, April 1989, Biochemistry, (US), V. De Luca et al.: "Molecular cloning and analysis of cDNA encoding a plant tryptophan decarboxylase: Comparison with animal dopa decarboxylases", pages 2582-2586 see the whole document</p> <p>-----</p>	1-14

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